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(54) DNA molecule encoding an aminopeptidase, and method of producing the aminopeptidase

(57) Disclosed are cDNA encoding a new aminopeptidase derived from germinated soybeans, a recombinant expression vector containing the DNA, a transformant obtained by the transformation with the expression vector, and a method of producing an aminopeptidase by culturing the transformed product.

According to the present invention, a cDNA can be obtained encoding the amino acid sequence of aminopeptidase GX suitable for producing a highly hydrolyzed product from a starting material containing a protein and peptide having a high acidic amino acid content. Using the cDNA, a recombinant aminopeptidase can be mass-produced with *E. coli* or the like. By using the cDNA, it is also possible to produce aminopeptidase GX by using a host other than *E. coli*. In addition, hydrolyzed products having high glutamic acid content and aspartic acid content and also excellent seasoning properties can be obtained from soybean protein by combining GX thus produced with protease D3 and leucine aminopeptidase DLAP.

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Description**BACKGROUND OF THE INVENTION**

5 [0001] The present invention relates to a DNA molecule encoding a new aminopeptidase derived from soybeans, a recombinant expression vector containing the DNA molecule, a transformant transformed with the recombinant expression vector, and a method of producing an aminopeptidase using the transformant.

[0002] Soybean protein is usually hydrolyzed into amino acids by the hydrolysis with an acid such as hydrochloric acid and sulfuric acid or with an existing proteases such as derived from a microorganism, e. g. an aspergillus.

10 [0003] However, when an acid proteolysis is used to obtain a proteolysis product of soybean protein which is useful as a natural seasoning, the reaction must be carried out at 100°C for one or two days. The reaction at such a high temperature for such a long time causes a problem of a high energy consumption. Although the hydrolysis of protein with an acid is easy, it has other problems of excess decomposition (degradation) and high salts content caused by the neutralization.

15 [0004] To solve these problems, it was suggested to hydrolyze the soybean protein with the existing protease under mild reaction conditions. In particular, the hydrolysis of the soybean protein into amino acids with the proteolytic enzymes (proteases) was expected to be a method which can be employed in place of the hydrolysis with acids by the chemical reaction, because the hydrolysis proceeds according to the biological reaction under mild reaction conditions.

[0005] However, storage protein in vegetables of the legume family is generally highly resistant to the existing proteases when the protein is native. Since existing proteases such as papain and subtilisin are typically endopeptidases, although they are capable of hydrolyzing protein into peptides, it is difficult to completely hydrolyze the protein into amino acids using only these proteases. In addition, the product thus obtained cannot actually be used as the seasoning liquid because it tastes bitter.

20 [0006] It was considered that the combination of endopeptidases and exopeptidases such as aminopeptidase and carboxypeptidase, which are also the enzymes for hydrolyzing peptides into amino acids, is effective for solving the above-described problems.

[0007] On the other hand, it was reported that leucine aminopeptidase and acidic carboxypeptidase are important for increasing in amount of free amino acids in the hydrolysis of soybean protein with an aspergillus in, for example, the brewing of soy sauce [Tadanobu Nakadai, "Shoken" Vol. 11, No. 2 (1985)]. However, as suggested in this report, the soy sauce still contains dipeptides and tripeptides containing acidic amino acids in the sequences thereof, and the difficulty of the hydrolysis of them was pointed out. The dipeptides and tripeptides also include peptides having glutamic acid or aspartic acid at the N-terminal thereof. The difficulty in the hydrolysis of the peptides indicates that the substrate specificity of the peptidase is low for these peptides. In addition to the problem of the difficult hydrolysis of peptides with the peptidase derived from the aspergillus in the brewing of soy sauce, commercially available peptidase preparations also have a problem that the hydrolysis activity of microbial enzymes, such as the enzyme from Aspergillus, is also low for dipeptides and tripeptides containing acidic amino acids.

[0008] Under these circumstances, the inventors tried to solve the above-described problems by using soybean cotyledons. Namely, the storage protein in soybean seeds is hydrolyzed into amino acids in a very short period of time in the course of the germination of the seeds. From this phenomenon, it is supposed that peptidases capable of easily hydrolyzing the poorly hydrolyzable peptides of the storage protein exist in the germinating soybeans. The inventors had found such peptidases (aminopeptidase GX and leucine aminopeptidases, which are capable of efficiently hydrolyzing acidic amino acid-containing peptides) in germinated soybean cotyledons, and succeeded in efficiently hydrolyzing the soybean protein [Japanese Patent Unexamined Published Application (hereinafter referred to as "JP-Kokai") No.9-294583].

45 [0009] However, it was difficult to obtain a large amount of soybean aminopeptidase GX from an extract from germinated soybean cotyledons because soybean aminopeptidase GX content of these cotyledons is only very low.

[0010] In one of the methods of solving the above-described problems, aminopeptidase genes are strongly expressed by a genetic recombination technique by using a system other than soybeans to obtain a large amount of the aminopeptidase. To carry out this method, it is essential to obtain the cDNA encoding the aminopeptidase and to analyze the DNA sequence thereof, to obtain an information of the whole amino acid sequence of the aminopeptidase.

50 [0011] It is also indispensable that DNA encoding the aminopeptidase is integrated into a suitable expression vector to obtain a transformant capable of producing the intended product in a large amount.

SUMMARY OF THE INVENTION

55 [0012] The present invention has been completed under these circumstances. The object of the present invention is to provide a technique for the efficient gene expression and mass-production of the aminopeptidase using gene recombination techniques, which is to be employed in place of the above-described natural method of isolating aminopepti-

dase from germinated soybean cotyledons from natural origin.

[0013] After intensive investigations made for the purpose of solving the above-described problems, the inventors succeeded in obtaining cDNA encoding aminopeptidase GX by screening cDNA library prepared from germinated soybean shoot mRNA by using rice plant EST as the probe which has an internal amino acid sequence highly homologous to that of aminopeptidase GX.

[0014] After further investigations, the inventors have succeeded in obtaining a transformant capable of forming the aminopeptidase from cDNA thus obtained, and then obtaining a protein having the aminopeptidase activity.

[0015] Namely, the present invention provides the following:

- 10 (1) A DNA molecule encoding a new aminopeptidase derived from germinated soybean cotyledons having an amino acid sequence of SEQ ID No:1 in the sequence list.
- (2) A recombinant DNA molecule containing the DNA molecule.
- (3) A transformant transformed with the recombinant DNA molecule.
- (4) A method of producing a protein having an aminopeptidase activity, which comprises culturing the transformant 15 to produce a protein having the aminopeptidase activity, and recovering the same.

[0016] After intensive investigations made for the purpose of solving the above-described problems, the inventors succeeded in obtaining cDNA encoding aminopeptidase GX by screening cDNA library prepared from germinated soybean shoot mRNA by using rice plant EST as the probe which has an internal amino acid sequence highly homologous to 20 that of aminopeptidase GX.

BRIEF DESCRIPTION OF THE DRAWING

[0017]

- 25 Fig. 1 shows the strategy for construction of plasmid pUCTRPGXN1-8.
- Fig. 2 shows the strategy for construction of GX expression plasmid pUCTRPGX1-8.
- Fig. 3 shows the strategy for construction of plasmid pUCTRPGXN2-1.
- Fig. 4 shows the strategy for construction of GX expression plasmid pUCTRPGX2-1.

DESCRIPTION OF THE PREFERRED EMBODIMENTS OF THE INVENTION

[0018] The present invention provides the followings:

- 35 (1) A DNA molecule encoding a new aminopeptidase derived from germinated soybean cotyledons having an amino acid sequence of SEQ ID NO:1 in the sequence list.
- (2) Variants of DNA molecule of (1) encoding the variants of the proteins having an amino acid sequence of SEQ ID NO: 1 in which one or more amino acid residues are inserted, added, deleted or replaced.
- (3) The DNA molecule of (1), which has a DNA sequence ranging from base No.22 to base No. 1428 in SEQ ID 40 NO:2 in the sequence list.
- (4) A recombinant DNA molecule containing the DNA molecule of any of (1) to (3).
- (5) A transformant transformed with the recombinant DNA molecule.
- (6) A method of producing a protein having an aminopeptidase activity, which comprises culturing the transformant to produce a protein having the aminopeptidase activity, and recovering the same.

45 [0019] The detailed description will be made on the present invention. Aminopeptidase GX will be referred to as "GX" hereinafter.

(1) DNA molecule of the present invention:

50 [0020] cDNA of the present invention can be obtained by, for example, an ordinary method wherein an oligo-DNA is synthesized on the basis of an already determined amino acid sequence of GX, gene segments are prepared by using mRNA extracted from germinated soybean cotyledons or another portion thereof as a template for RT-PCR method, and cDNA for GX is cloned by the hybridization using the gene segments as probes, from cDNA library prepared from 55 mRNA of the cotyledons or another portion of the germinated soybeans as the template.

[0021] In another possible method, a DNA sequence highly homologous to that encoding the already determined amino acid sequence of GX is retrieved from a suitable DNA data base such as DDBJ, EMBL or GenBank, a cDNA library was prepared using mRNA extracted from the cotyledons or another portion of the germinated soybeans as a

template, and the cDNA library is screened to obtain cDNA of GX using the DNA segment of corresponding sequence as the probe.

[0022] The kind of the soybeans to be germinated for the extraction of mRNA from GX is not limited. Namely, the cultivating area and variety of soybeans are not limited; and commercially available soybeans, those used as a material for squeezing soybean oil, etc. are usable. Further, the method of the germination, culture conditions, stage (germinated or non-germinated) and duration of the germination of them are not limited. It is preferred, however, to use germinated soybeans obtained by growing them for 7 to 10 days, after soaking soybean seeds in water.

[0023] The probe for cDNA can also be prepared by RT-PCR method or the like on the basis of an already determined amino acid sequence by using mRNA extracted from the cotyledons or another portion of germinated soybean cotyledons; or by chemically synthesis on the basis of the amino acid sequence. A DNA sequence highly homologous to the amino acid sequence is usable as the probe, regardless of the origin of the DNA sequence and the function of the gene product. Namely, the DNA sequence is not limited to that of a known aminopeptidase; the gene product having an unknown function is also usable; and EST (Expression Sequence Tag) is also usable. In the Examples given below, EST derived from rice plant roots was used as the probe. The cDNA library can be produced by an ordinary method.

[0024] Thus, DNA molecule encoding the new aminopeptidase derived from germinated soybean cotyledons and having an amino acid sequence of SEQ ID NO:1 in the sequence list can be obtained. DNA molecule of the present invention may be the one which encodes a protein having an amino acid sequence of SEQ ID No: 1 in the sequence list, in which one or more amino acid residues are inserted, added, deleted or replaced, so far as the protein encoded by the DNA molecule has the aminopeptidase activity.

[0025] After further investigations, the inventors have succeeded in obtaining a transformant capable of forming the aminopeptidase from cDNA thus obtained, and then obtaining a protein having the aminopeptidase activity.

(2) Recombinant DNA molecule of the present invention:

[0026] cDNA thus obtained is integrated into an expression vector to obtain a recombinant DNA molecule. The vector to be used is not particularly limited. The vector may be the one capable of autonomously replicating in the host cells or the one capable of being inserted in the chromosome in more than one copy. The vector must have an insertion site in which the above-described DNA, i. e. GX gene, can be inserted and further a region which allows the inserted DNA to be expressed in the host cells.

[0027] The GX genes to be inserted into the vector are not limited to only cDNA but they also include DNA fragments designed so as to code the amino acid sequence of GX deduced from cDNA. The genes deduced from such an amino acid sequence can be easily synthesized by ligating an oligonucleotide synthesized with an automatic DNA synthesizing machine after the annealing.

[0028] In another method, GX is expressed and produced in the form of a fused protein associated with a heterologous protein. For example, GX can be produced in Escherichia coli (E. coli) in the form of a fused protein, linked to glutathione-S-transferase using pGEX system (a product of Amersham Pharmacia Biotech. Co.) or the like.

[0029] As promoters for expressing the GX genes, strong promoters usually used for the expression of heterologous proteins can be used. A terminator can be introduced into a downstream of the GX gene. The examples of promoters include, for example, trp, tac, lac, trc, λPL and T7, and the terminators include, for example, tpA, lpp and T4.

[0030] For making the translation more efficient, the variety and number of SD sequence, and the base composition, sequence and length in the region between the SD sequence and the initiation codon are preferably optimized for the expression of GX gene.

[0031] The region between the promoter and the translation-initiating point, required for the expression of GX, can be prepared by a well-known PCR method or chemical synthesis method. An example of the sequences is shown in SEQ ID NO:3.

[0032] The recombinant DNA molecule of the present invention can be obtained by inserting the above-described GX gene-containing DNA fragment into a well-known expression vector selected depending on the intended expression system. The expression vector used herein is preferably a multi copy vector.

[0033] Known vectors usable for the preparation of the recombinant DNA molecule of the present invention are pUC18, pHSG299, etc. An example of the recombinant DNA molecule of the present invention is pUCTRPGX1-8, which is obtained by integrating DNA molecule of the present invention into pUC18.

(3) Transformant of the present invention:

[0034] The description will be made on various transformants obtained by the introduction of the above-described recombinant DNA molecule.

[0035] The cells which can be converted into the transformants are those of bacteria, such as E. coli or the like. Examples of E. coli strain include JM 109 strain (recA, endA1, gyrA96, thi, hsdR17, supE44,r elA1, and Δ(lac-proAB)/F'

[traD36, proAB+, lacIq and LacZΔM15]).

[0036] The other cells which can be converted into the transformants are those of *Bacillus subtilis*, yeast, *Aspergillus*, etc. It is possible to produce GX into a medium, taking advantage of the protein-secreting properties of them. In addition to the above-described microorganisms, cultured cells such as those of silk worms are also usable.

5 [0037] Then the recombinant vector obtained as described above is introduced into a host cell to obtain the transformant. The recombinant vector can be introduced into the host cell by various conventional methods, for example, competent cell method, protoplast method, calcium phosphate coprecipitation method, electroporation method, microinjection method and liposome fusion method.

10 (4) Method for producing the aminopeptidase of the invention:

[0038] The transformant thus obtained is cultured to produce GX in the culture mixture. GX is isolated by a well-known method and, if necessary, purified to obtain the intended enzyme.

15 [0039] When *E. coli* is used as a host, it is possible that GX gene product is obtained as an inert GX association product, i. e. protein inclusion body, and then this inclusion body is activated by a suitable method. After the re-activation, the active protein can be separated and purified by a well-known method to obtain the intended enzyme.

20 [0040] The media for culturing the transformant are well known. For example, for culturing *E. coli*, a nutrient medium such as LB medium or a minimal medium such as M9 medium is used with the addition of a carbon source, a nitrogen source, a vitamin source, etc. The transformant is cultured at a temperature of usually 16 to 42°C, preferably 25 to 37°C, for 5 to 168 hours, preferably 8 to 72 hours. The culture conditions vary depending on the host. Both shaking culture and standing culture are possible. If necessary, the medium may be stirred or aerated. When a inducible promoter is used for expressing GX, a promoter inducer can be added to the medium.

25 [0041] GX can be isolated and purified from the extract of transformant by a well-known method such as salting-out method, isoelectric precipitation method or solvent precipitation method; a method wherein a difference in the molecular weight is utilized such as dialysis, ultrafiltration or gel filtration; a method wherein a specific affinity is utilized such as ion exchange chromatography; a method wherein a difference in the hydrophobicity is utilized such as hydrophobic chromatography or reversed phase chromatography; affinity chromatography; SDS polyacrylamide electrophoresis; or isoelectric focusing method. GX can be purified by a combination of these methods.

30 [0042] According to the present invention, a cDNA can be obtained encoding the amino acid sequence of aminopeptidase GX suitable for producing a highly hydrolyzed product from a starting material containing a protein and peptide having a high acidic amino acid content. Using the cDNA, a recombinant aminopeptidase can be mass-produced with *E. coli* or the like. By using the cDNA, it is also possible to produce aminopeptidase GX by using a host other than *E. coli*. In addition, hydrolyzed products having high glutamic acid content and aspartic acid content and also excellent seasoning properties can be obtained from soybean protein by combining GX thus produced with commercially available 35 proteases such as Flavourzyme™ (manufactured by Novo Nordisk A/S) and Protease M™ (manufactured by Amano Pharmaceutical Co., Ltd.).

protease D3 and leucine aminopeptidase DLAP.

40 EXAMPLES

[0043] The following Examples is provided to only illustrate the present invention and not to limit the scope of the present invention to these examples.

45 Example 1: Cloning of GX cDNA:

[0044] In this Example, the present invention will be described on (1) determination of internal amino acid sequence of GX, (2) search for a DNA sequence having a high homology to that of GX, (3) analysis of GX expression site in soybeans and (4) screening of cDNA library of germinated soybean shoots using rice plant EST R2219_2A as the probe.

50 (1) Determination of internal amino acid sequence of GX:

[0045] GX protein obtained from germinated soybean cotyledons was reduced, carboxymethylated and treated with lysyl endopeptidase (EC. 3. 4. 21. 50 Wako Pure Chemical Industries, Ltd.). Peptide fragments thus obtained were taken with μRPC C2/C18 SC2.1/10 column (a product of Pharmacia Aktiebolag). The amino acid sequences of 10 portions of the fragment in total was successfully determined using a protein sequencer, after analyzing the amino acid sequence of each fragments.

(2) Search for DNA sequence having high homology to GX:

[0046] The amino acid sequences determined as described above were subjected to the homology retrieval of DDBJ (DNA DATA BANK of JAPAN) to find EST R2219_2A derived from rice plant roots and having a high homology to peptide fragment No. 8 of SWQ ID NO:4 in the sequence list. It is highly possible that R2219_2A of rice plant thus obtained is encoding GX homologue of rice plant. Then, the cloning of soybean GX was tried by using R2219_2A of rice plant as the probe. Before the cloning, the investigations were made to find a organ of the soybeans in which the expression was remarkable.

10 (3) Analysis of GX expression site in soybeans:

[0047] Fragments of rice plant R2219_2Ac DNA to be used as the probes were obtained by RT-PCR method as described below.

[0048] A fragment of R2219_2AcDNA was amplified by RT-PCR method using poly(A) RNA of rice root as the template. The primers for the amplification of R2219_2A were R2219_2AU (SEQ ID NO:5 in the sequence list) as the sense primer and R2219_2AD (SEQ ID NO: 6 in the sequence list) as the antisense primer. The amplified region was found to contain the region corresponding to peptide No. 8.

[0049] For RT-PCR reaction, Takara RNA PCR kit was used. The PCR conditions were: 94°C for 5 minutes, then 55°C for 1 minute and 72°C for one minute in one cycle; and then 94°C for 1 minute, then 55°C for 1 minute and 72°C for one 20 minute in 25 cycles.

[0050] Then, the Northern hybridization was carried out to elucidate whether soybean RNA had a sequence homologous to that of rice plant R2219_2A using above-described rice plant 2219_2Ac DNA.

[0051] The Northern blotting of the total RNA extracted from the respective organ of the soybeans was carried out by using R2219_2Ac DNA obtained as described above. The organ of the soybeans used were day three and day seven 25 cotyledons after the germination, seven day shoots after the germination, immature cotyledons, pods and leaves.

[0052] After the hybridization at 55°C or 60°C followed by the stringent washing with 0.5xSSC, 0.1 % SDS at a temperature equal to that of the hybridization, day seven shoots after the germination, produced a clear signal. The size thereof was calculated to be about 1.5 kbp.

[0053] From this result, it was concluded that the soybean shoots had mRNA having some homology to that of rice 30 plant R2219_2A, and it was strongly expected to be GX mRNA. The above experiments indicated that GX cDNA would be most likely obtained by screening cDNA library prepared from mRNA of soybean shoots on the seventh day after the germination under the hybridization condition of 60°C by using the rice plant R2219_2AcDNA as the probe.

(4) Screening for cDNA library derived from germinated soybean shoots by using rice plant EST as the probe:

[0054] cDNA library 7.2×10^4 pfu prepared from polyA RNA of soybean seven day shoots after the germination was screened by an ordinary method under the above-described conditions to obtain seven hybridizing clones. The DNA sequence of each clone was determined to find a clone containing the full length GX DNA sequence of SEQ ID NO: 2, which encodes the polypeptide comprising amino acids of 487 residues of SEQ ID NO: 1 and which contains GX internal amino acid sequence of SEQ ID NO: 4.

[0055] It was considered that this DNA sequence was that of the intended GX cDNA because it encoded all of the 10 regions of GX internal amino acid sequences determined as stated above. Then, it was tried to produce GX protein by E. coli using this cDNA.

45 Example 2 :Production of GX by E coli:

[0056] GX cDNA obtained in Example 1 was integrated into an expression vector which functions in E. coli, and transformants containing the expression plasmid were cultured. GX activity was detected in the cells. In addition to the sequence of the obtained GX cDNA, expression plasmids were also prepared for those in which the codons were 50 changed in four residues ranging from Ala (the second residue from the N terminal) to Leu (the fifth residue), i.e. Ala, Ala, Lys and Leu, taking the codon usage for E. coli into consideration. The expression levels of them were compared with one another.

[0057] In this Example, the present invention will be illustrated on (1) the construction of expression plasmid, (2) the preparation of E. coli transformant using the expression plasmid and the culture thereof and (3) the determination of GX 55 activity.

(1) Construction of expression plasmid:

(1) Construction of expression plasmid having the unmodified sequence of GX cDNA:

5 [0058] trp Promoter the transcription of which can be easily induced by the lacking of tryptophan in the medium was used as the promoter for transcribing GX genes. trp Promoter was used for plasmid pTTG-22 (JP-Kokai No.6-225775) which highly expressed Pagrus major transglutaminase (TG) genes. The sequence of the upstream of the Pagrus major TG gene was designed so that heterologous protein would be highly expressed in E. coli. Plasmid pUCTRPM TG(+)-D2 (EP-A- 0889133) which highly expressed transglutaminase (MTG) genes derived from microorganisms had the upper stream sequence (SEQ ID NO: 3) containing trp promoter of the expression plasmid of Pagrus major TG. By the further integration into multi-copy plasmid pUC19, MTG was highly expressed.

10 [0059] DNA fragment was linked to trp promoter to the upstream region of GX cDNA using PCR. At first, as shown in Fig. 1, the region (SEQ ID NO: 3) containing trp promoter of MTG expression plasmid pUCTRPM TG(+)-D2 and the partial region of GX cDNA were amplified by PCR. The primers for amplifying trp promoter were TRP-N2 (SEQ ID NO. 7) and TRP-C2 (SEQ ID NO:8); the primers for amplifying GX were GX-N1 (SEQ ID NO:9) and GX-C (DEQ ID NO:10); TRP-N2 and GX-N1 were sense primers; and TRP-C2 and GX-C are antisense primers. GX-N1 was designed to add 11 bases DNA sequence which would be used for linking GX to the trp promoter containing fragment, immediately upstream the initiation codon of GX. This sequence is complementary to the sequence in TRP-C2.

15 [0060] PCR was conducted by using plasmid pUCTRPM TG(+)-D2 and primers TRP-N2 and TRP-C2; and plasmid pR2219i containing the full length GX cDNA and primers GX-N1 and GX-C. The PCR reaction conditions were: 94°C for 2 minutes in one cycle; and 94°C for 30 seconds, then 50°C for 5 seconds and 72°C for 30 seconds in 25 cycles. The PCR products were treated with phenol/chloroform and then precipitated with ethanol. Each precipitant was dissolved in 100 µL of dH₂O.

20 [0061] Aliquot (1 µL) was taken from each of the PCR products and they were mixed. After the heat denaturation at 94°C for 10 minutes, PCR was carried out by using primers TRP-N2 and GX-C for 25 cycles. The conditions in each cycle comprised 94°C for 30 seconds, then 55°C for 5 seconds and then 72°C for 1 minute.

25 [0062] The second PCR product was extracted with phenol/chloroform. After the precipitation with ethanol, the product was digested with EcoRI and KpnI and then subcloned into pUC18 to obtain pUCTRPGXN1-8 (Fig. 1). The sequence was confirmed.

30 [0063] Then, C-terminal region of GX gene contained in pR2219i was excised using KpnI and XbaI and subcloned into the above-described pUCTRPGXN1-8 to obtain GX expression plasmid pUCTRPGX1-8 driven by trp promoter(Fig. 2).

(2) Construction of GX expression plasmid with modified codons:

35 [0064] The plasmid was constructed in the same manner as that of above-described method (1) except that GX-N2 (SEQ ID NO:11) was used as the sense primer for the amplification of N-terminal segment of GX cDNA by PCR. In GX-N2, codons corresponding to the second to the fifth residues from the N-terminal, i. e. codons corresponding to Ala, Ala, Lys and Leu, were changed from GCG GCG AAG CTA to GCT GCT AAA CTG.

40 [0065] PCR was conducted by using plasmid pUCTRPM TG(+)-D2 and primers TRP-N2 and TRP-C2; or plasmid pR2219i and primers GX-N2 and GX-C. The PCR reaction conditions were: 94°C for 2 minutes in one cycle; and 94°C for 30 seconds, then 50°C for 5 seconds and 72°C for 30 seconds in 25 cycles. The PCR products were treated with phenol/chloroform and then precipitated with ethanol. Each product was dissolved in 100 µL of dH₂O.

45 [0066] Aliquot (1 µL) was taken from each of the PCR products and they were mixed. After heat denaturation at 94°C for 10 minutes, PCR was carried out by using primers TRP-N2 and GX-C for 25 cycles. Conditions in each cycle comprised 94°C for 30 seconds, then 55°C for 5 seconds and then 72°C for 1 minute.

50 [0067] The second PCR product was extracted with phenol/chloroform. After the precipitation with ethanol, the product was digested with EcoRI and KpnI and then subcloned into pUC18 to obtain pUCTRPGXN2-1 (Fig. 3). The sequence was confirmed.

55 [0068] Then, C-terminal region of GX gene contained in pR2219i was excised by using KpnI and XbaI and subcloned into the above-described pUCTRPGXN2-1 to obtain GX expression plasmid pUCTRPGX2-1 driven by trp promoter (Fig. 4).

(2) Preparation and culture of of E. coli transformant using the expression plasmid pUCTRPGX2-1:

55 [0069] pUCTRPGX1-8, pUCTRPGX2-1 and pUC19 were introduced into E. coli JM109 by the competent cell method, and the transformant was selected in an agar medium containing 150 µg/mL of ampicillin. E. coli transformed with pUCTRPGX2-1 was named "AJ13564" and deposited in National Institute of Bioscience and Human-Technology

Agency of Industrial Science and Technology as FERM P-17131, which was relegated to international deposition in the same institute as FERM BP-7027 under the Budapest Treaty on February 14, 2000. Each transformant was inoculated into 2 × YT medium containing 150 µg/mL of ampicillin and cultured at 37°C for 5 hours. 1 mL of the preculture liquid thus obtained was transferred into 50 mL of M9-Casamino acid medium containing 150 µg/mL of ampicillin to conduct the main culture. The main culture was conducted at 37°C for 18 hours.

[0070] After the completion of the culture, the cells collected by the centrifugation were suspended in a cell-homogenizing buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA] and the sonication was conducted with microchips of Branson MODEL-Sonifier 250 under conditions comprising output control 7, duty cycle 50 % and about 10 minutes. The liquid thus obtained was centrifuged at 12,000 rpm for 10 minutes. The supernatant liquid was used as the soluble fraction in the cells, and the precipitates were used as the insoluble fraction. After the SDS-polyacrylamide gel electrophoresis, the expression of a protein having a molecular weight equal to that of GX was recognized in the pUCTRPGX1-8/JM109-soluble fraction, and in the pUCTRPGX2-1/JM109-soluble fraction and -insoluble fraction. A particularly high expression was observed in the pUCTRPGX2-1/JM109-soluble fraction in which the codon had been modified. This fact clearly shows the effects obtained by changing the codon. A sufficiently high expression was obtained in the production medium even without the addition of 3-β-indoleacrylic acid.

(3) Determination of GX activity:

[0071] The GX activity was determined on the basis of the increase in the amount of free glutamic acid due to the hydrolysis of dipeptide Glu-Glu. An enzyme solution was added to an activity determining solution containing 50 mM of HEPES (pH 8.0) and 5 mM of Glu-Glu. After carrying out the reaction at 37°C for 5 to 10 minutes, acetic acid was added to the reaction mixture to the final concentration of 2 % and thereby to terminate the reaction. The quantity of free glutamic acid was determined with a glutamic acid assay kit (a product of Seikagaku Kogyo). An activity for producing 1 µmol of glutamic acid in one minute was determined to be one unit.

[0072] GX activities of the soluble fractions of pUCTRPGX1-8/JM109, pUCTRPGX2-1/JM109 and pUC19/JM109, namely, the supernatant liquid containing the cell debris, were determined to calculate the specific activities. The specific activities obtained were 0.91 U/mg for pUCTRPGX1-8/JM109, 2.88 U/mg for pUCTRPGX2-1/JM109 and 0.04 U/mg for pUC19/JM109. It was thus confirmed that GX protein accumulated in the cells had the activity. It was also confirmed that by modifying the codon, about three times as much amount of GX protein was accumulated.

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Sequence Listing

5 <110> Ajinomoto Co., Inc.

10 <120> DNA encoding new aminopeptidase, and method of producing the
aminopeptidase

15 <130> OP00010

20 <150> JP 11-68255

25 <151> 1999-03-15

30 <160> 11

35 <210> 1

40 <211> 487

45 <212> PRT

50 <213> Glycine max

<400> 1

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40 1 5 10 15

Phe Leu Asn Ala Ser Pro Thr Ala Phe His Ala Val Asp Glu Ala Lys
45 20 25 30

Arg Arg Leu Arg Ser Ala Gly Tyr His Gln Leu Ser Glu Arg Glu Val
50 35 40 45

Trp Glu Leu Gln Pro Gly Asn Lys Tyr Phe Phe Thr Arg Asn His Ser

55

	50	55	60
5	Thr Ile Val Ala Phe Ala Ile Gly Lys Lys Tyr Val Ala Gly Asn Gly		
	65	70	75
10	Phe Tyr Ile Ile Gly Ala His Thr Asp Ser Pro Cys Leu Lys Leu Lys		80
	85	90	95
15	Pro Val Thr Lys Val Val Lys Ala Gly Ile Leu Glu Val Gly Val Gln		
	100	105	110
20	Thr Tyr Gly Gly Leu Trp His Thr Trp Phe Asp Arg Asp Leu Thr		
	115	120	125
25	Val Ala Gly Arg Val Ile Val Arg Glu Glu Asn Ala Gly Ser Val Ser		
	130	135	140
30	Tyr Ser His Arg Leu Val Arg Ile Glu Glu Pro Ile Met Arg Ile Pro		
	145	150	155
35	Thr Leu Ala Ile His Leu Asp Lys Thr Val Asn Asp Gly Phe Lys Phe		
	165	170	175
40	Asn Asn Glu Asn His Leu Ile Pro Ile Leu Ala Thr Ser Leu Lys Gly		
	180	185	190
45	Glu Leu Asn Lys Val Ser Ser Glu Asn Gly Pro Val Glu Ser Gly Asn		
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50	Gln Thr Asp Gly Lys Lys Ala Asn Asp Lys Thr Gly Thr Ser Asn Thr		
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55	Lys His His Leu Leu Leu Leu Gln Leu Leu Ala Ser Lys Leu Gly Cys		
	225	230	235
60	Glu Pro Asp Asp Ile Cys Asp Phe Glu Leu Gln Ala Cys Asp Thr Gln		240
	245	250	255
65	Pro Ser Thr Ile Ala Gly Ala Ala Lys Glu Phe Ile Phe Ser Gly Arg		

	260	265	270
5	Leu Asp Asn Leu Cys Met Ser Phe Cys Ser Leu Lys Ala Leu Ile Asp		
	275	280	285
	Ala Thr Ser Ser Asp Ser Ser Leu Glu Glu Glu Ser Gly Val Arg Met		
10	290	295	300
	Val Ala Leu Phe Asp His Glu Glu Val Gly Ser Asn Ser Ala Gln Gly		
15	305	310	315
	Ala Gly Ser Pro Val Met Leu Asn Ala Val Thr Arg Val Thr Asn Ser		
	325	330	335
20	Phe Ser Ser Asn Pro Asn Leu Leu Glu Lys Ala Ala Gln Leu Ser Tyr		
	340	345	350
25	Leu Val Ser Ala Asp Met Ala His Ala Leu His Pro Asn Tyr Met Asp		
	355	360	365
	Lys His Glu Ala Asn His Gln Pro Lys Leu His Gly Gly Leu Val Ile		
30	370	375	380
	Lys Thr Asn Ala Ser Gln Arg Tyr Ala Thr Asn Val Val Thr Ser Phe		
35	385	390	395
	Ile Phe Arg Glu Ile Ala Ser Lys His Lys Leu Pro Val Gln Asp Phe		
	405	410	415
40	Val Val Arg Asn Asp Met Ser Cys Gly Ser Thr Ile Gly Pro Ile Leu		
	420	425	430
45	Ala Ser Gly Val Gly Ile Arg Thr Val Asp Val Gly Ala Pro Gln Leu		
	435	440	445
	Ser Met His Ser Ile Arg Glu Ile Cys Ala Val Asp Asp Val Lys Tyr		
50	450	455	460
	Ser Tyr Glu His Phe Lys Ala Phe Tyr Gln Glu Phe Ser His Val Asp		
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	Met Ala Ala Lys Leu Asp Thr His Ala Val				
	1	5	10		
35	gct tcc gat ctg atc gac ttc ctc aac gct tct cca acg gct ttc cac				99
	Ala Ser Asp Leu Ile Asp Phe Leu Asn Ala Ser Pro Thr Ala Phe His				
	15	20	25		
40	gcc gtc gac gag gca aag agg cgt ttg cgt agc gcg ggg tac cac caa				147
	Ala Val Asp Glu Ala Lys Arg Arg Leu Arg Ser Ala Gly Tyr His Gln				
	30	35	40		
45	ctc tct gag agg gaa gtg tgg gaa ctg caa ccg ggc aac aag tac ttc				195
	Leu Ser Glu Arg Glu Val Trp Glu Leu Gln Pro Gly Asn Lys Tyr Phe				
	45	50	55		

55

	ttc acc aga aat cac tcc acc atc gtc gcc ttc gcc atc ggc aaa aag		243
5	Phe Thr Arg Asn His Ser Thr Ile Val Ala Phe Ala Ile Gly Lys Lys		
	60	65	70
	tac gtt gct gga aat gga ttc tac ata att ggg gct cac acg gat agt		291
10	Tyr Val Ala Gly Asn Gly Phe Tyr Ile Ile Gly Ala His Thr Asp Ser		
	75	80	85
15	cct tgt ctc aaa ctc aag cct gtc acc aag gtt gtt aag gct ggg att		339
	Pro Cys Leu Lys Leu Lys Pro Val Thr Lys Val Val Lys Ala Gly Ile		
	95	100	105
20	ttg gag gtt ggt gtc caa acc tat gga ggt ggt ctg tgg cac aca tgg		387
	Leu Glu Val Gly Val Gln Thr Tyr Gly Gly Leu Trp His Thr Trp		
	110	115	120
25	ttt gat cga gac ttg act gtg gcg ggg agg gtc atc gtg cgg gaa gag		435
	Phe Asp Arg Asp Leu Thr Val Ala Gly Arg Val Ile Val Arg Glu Glu		
30	125	130	135
	aat gct ggt tct gtt tcg tac tca cat cgc ctt gtt aga att gag gaa		483
35	Asn Ala Gly Ser Val Ser Tyr Ser His Arg Leu Val Arg Ile Glu Glu		
	140	145	150
	cct ata atg cga ata ccg act ttg gca att cac ttg gac aag act gtt		531
40	Pro Ile Met Arg Ile Pro Thr Leu Ala Ile His Leu Asp Lys Thr Val		
	155	160	165
45	aat gat gga ttc aaa ttt aac aac gag aat cac ctt att ccc atc ttg		579
	Asn Asp Gly Phe Lys Phe Asn Asn Glu Asn His Leu Ile Pro Ile Leu		
	175	180	185
50	gca aca tcg ctg aag ggt gag ctc aat aaa gtg tcc tct gaa aat ggt		627
	Ala Thr Ser Leu Lys Gly Glu Leu Asn Lys Val Ser Ser Glu Asn Gly		

	190	195	200	
5	cct gtt gaa agt gga aat cag acc gat gga aag aaa gca aat gat aaa			675
	Pro Val Glu Ser Gly Asn Gln Thr Asp Gly Lys Lys Ala Asn Asp Lys			
	205	210	215	
10	aca ggc acc agc aat acg aag cat cac ctt ctt ctt cta cag ttg ctt			723
	Thr Gly Thr Ser Asn Thr Lys His His Leu Leu Leu Leu Gln Leu Leu			
	220	225	230	
15	gca agc aag ctt ggg tgt gaa cca gat gac ata tgt gat ttt gaa ttg			771
	Ala Ser Lys Leu Gly Cys Glu Pro Asp Asp Ile Cys Asp Phe Glu Leu			
20	235	240	245	250
	caa gct tgc gat aca caa cca agt act att gct gga gct gca aag gaa			819
	Gln Ala Cys Asp Thr Gln Pro Ser Thr Ile Ala Gly Ala Ala Lys Glu			
	255	260	265	
25	ttc att ttt tca gga cgg ctt gat aat ctc tgc atg tca ttt tgc tcg			867
	Phe Ile Phe Ser Gly Arg Leu Asp Asn Leu Cys Met Ser Phe Cys Ser			
30	270	275	280	
	ctg aag gca tta ata gat gct aca tct tct gac agc agt ctt gag gaa			915
	Leu Lys Ala Leu Ile Asp Ala Thr Ser Ser Asp Ser Ser Leu Glu Glu			
	285	290	295	
35	gag tca ggt gtt aga atg gtg gct tta ttt gac cat gag gaa gtt gga			963
	Glu Ser Gly Val Arg Met Val Ala Leu Phe Asp His Glu Glu Val Gly			
40	300	305	310	
	tct aac tct gcc caa gga gct ggc tct cct gtt atg cta aat gct gtg			1011
	Ser Asn Ser Ala Gln Gly Ala Gly Ser Pro Val Met Leu Asn Ala Val			
45	315	320	325	330
	act agg gtt acc aat tcc ttc agc tcc aat ccc aac ctt ctg gag aaa			1059

	Thr Arg Val Thr Asn Ser Phe Ser Ser Asn Pro Asn Leu Leu Glu Lys			
5	335	340	345	
	gca gca caa tta agc tac ctt gta tct gcc gac atg gca cat gca cta		1107	
	Ala Ala Gln Leu Ser Tyr Leu Val Ser Ala Asp Met Ala His Ala Leu			
10	350	355	360	
	cac cca aat tac atg gac aag cat gaa gca aac cat cag ccc aaa cta		1155	
15	His Pro Asn Tyr Met Asp Lys His Glu Ala Asn His Gln Pro Lys Leu			
	365	370	375	
	cat gga gga ctt gtc att aaa acc aat gca agc caa cgc tat gca acc		1203	
20	His Gly Gly Leu Val Ile Lys Thr Asn Ala Ser Gln Arg Tyr Ala Thr			
	380	385	390	
25	aat gtt gtc aca tcc ttc ata ttc agg gag ata gca tca aaa cat aaa		1251	
	Asn Val Val Thr Ser Phe Ile Phe Arg Glu Ile Ala Ser Lys His Lys			
	395	400	405	410
30	ctt ccc gtt cag gac ttt gtg gtg cgc aat gac atg tca tgt ggt tca		1299	
	Leu Pro Val Gln Asp Phe Val Val Arg Asn Asp Met Ser Cys Gly Ser			
	415	420	425	
35	acc att ggt cct att ctt gct agt ggc gta ggt att cgc act gtt gat		1347	
	Thr Ile Gly Pro Ile Leu Ala Ser Gly Val Gly Ile Arg Thr Val Asp			
40	430	435	440	
	gta ggt gca ccg cag ttg tca atg cat agc ata cga gaa att tgt gct		1395	
	Val Gly Ala Pro Gln Leu Ser Met His Ser Ile Arg Glu Ile Cys Ala			
45	445	450	455	
	gtt gat gat gtg aag tat tca tat gag cac ttc aaa gca ttt tac caa		1443	
50	Val Asp Asp Val Lys Tyr Ser Tyr Glu His Phe Lys Ala Phe Tyr Gln			
	460	465	470	

55

gaa ttc tct cat gtt gat ggt aag atg gtc gtg gat ata tag gaatatctc 1494

5 Glu Phe Ser His Val Asp Gly Lys Met Val Val Asp Ile

475 480 485

taatcaccaa atcctcatta atccttgct ctagaagctg ttgctgaaat ggtcggttt 1554

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cgtccctgaat gaaatattag caactaaaaa aaaaaaaaaaaa aaa 1657

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gacagggttc ccgactggaa agcgggcagt gagcgcaacg caattaaatgt gagttagctc 120

40 actcattagg caccccgaggc tttacacttt atgcttccgg atcgtatgtt gtgtgaaatt 180

gtgagcggat aacaatttca cacagggaaac agctatgacc atgattacgc caagcttgca 240

tgcctcgagg tcgcccatttc gtcttcaaga attccctgt tgacaattaa tcatcgaaact 300

45 agttaacttag tacgcaagtt cacgtaaaaa gggtatcgat tagtaaggag gtttaaa 357

50 <210> 4

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5 <212> PRT

10 <213> Glycine max

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20 Ser Ala Gly Tyr His Gln Leu Ser Glu Arg Glu Val Trp Glu Leu Gln Pro Gly

25 1

30 5

35 10

40 15

45 Asn Lys

50 20

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65 <212> DNA

70 <213> Artificial Sequence

75 30

80 <220>

85 <223> Description of Artificial Sequence : primer

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95 <400> 5

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105 25

110 40

115 <210> 6

120 <211> 25

125 <212> DNA

130 <213> Artificial Sequence

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140 <220>

145 55

<223> Description of Artificial Sequence : primer

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caggctttag cttcagggat ggact 25
10

15 <210> 7
<211> 20
<212> DNA
<213> Artificial Sequence
20

25 <220>
<223> Description of Artificial Sequence : primer

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35 <210> 8
<211> 27
<212> DNA
40 <213> Artificial Sequence

45 <220>
<223> Description of Artificial Sequence : primer

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5 <210> 9

10 <211> 32

<212> DNA

15 <213> Artificial Sequence

<220>

20 <223> Description of Artificial Sequence : primer

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ggaggtttaa aatggcgccg aagctagaca cc

32

30

<210> 10

35 <211> 21

<212> DNA

40 <213> Artificial Sequence

30

<220>

35 <223> Description of Artificial Sequence : primer

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21

45 <210> 11

<211> 32

50 <212> DNA

<213> Artificial Sequence

55

<220>

<223> Description of Artificial Sequence : primer

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<400> 11

10 ggaggttaa aatggctgct aaactggaca cc 32

15

Claims

1. A DNA molecule encoding an aminopeptidase derived from germinating soybean cotyledon and having an amino acid sequence of SEQ ID NO:1.
- 20 2. A DNA molecule encoding a variant of an aminopeptidase derived from germinating soybean cotyledon and having an amino acid sequence of SEQ ID NO:1, wherein one or more amino acid residues are inserted into, added to, deleted from or substituted in the amino acid sequence of SEQ ID NO:1.
- 25 3. The DNA molecule of claim 1, which has the sequence of nucleotide No.22 to No.1482 of SEQ ID NO:2.
4. A DNA molecule which can hybridize to the DNA molecule having the sequence of SEQ ID NO:2 in stringent conditions and encodes a protein having an aminopeptidase activity.
- 30 5. The DNA molecule of claim 1, wherein codon usage is optimized for Escherichia coli.
6. The DNA molecule of claim 5, wherein the codon for Ala²-Ala³-Lys⁴-Leu⁵ is GCTGCTAAACTG.
7. A recombinant DNA molecule comprising the DNA molecule of claim 1.
- 35 8. A recombinant DNA molecule comprising the DNA molecule of claim 2.
9. A recombinant DNA molecule comprising the DNA molecule of claim 4.
- 40 10. The recombinant DNA molecule of claim 7, which is a high expression vector.
11. The recombinant DNA molecule of claim 7, wherein a trp promoter is functionally linked to the DNA molecule of claim 1.
- 45 12. The recombinant DNA molecule of claim 7, which is a high copy vector.
13. A host transformed with the DNA molecule of claim 1.
14. The transformed host of claim 13, which is a prokaryote.
- 50 15. The transformed host of claim 13, which is a eukaryote.
16. The transformed host of claim 14, which is Escherichia coli.
- 55 17. An Escherichia coli cell transformed with the DNA molecule of claim 5.
18. The Escherichia coli cell of claim 17, deposited as FERM BP-7027

19. A method of producing a protein having aminopeptidase activity, which comprises the steps of

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**(i) culturing the transformed host of claim 13,
(ii) expressing a protein having the aminopeptidase activity, and
(iii) recovering the protein.**

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FIG. 1

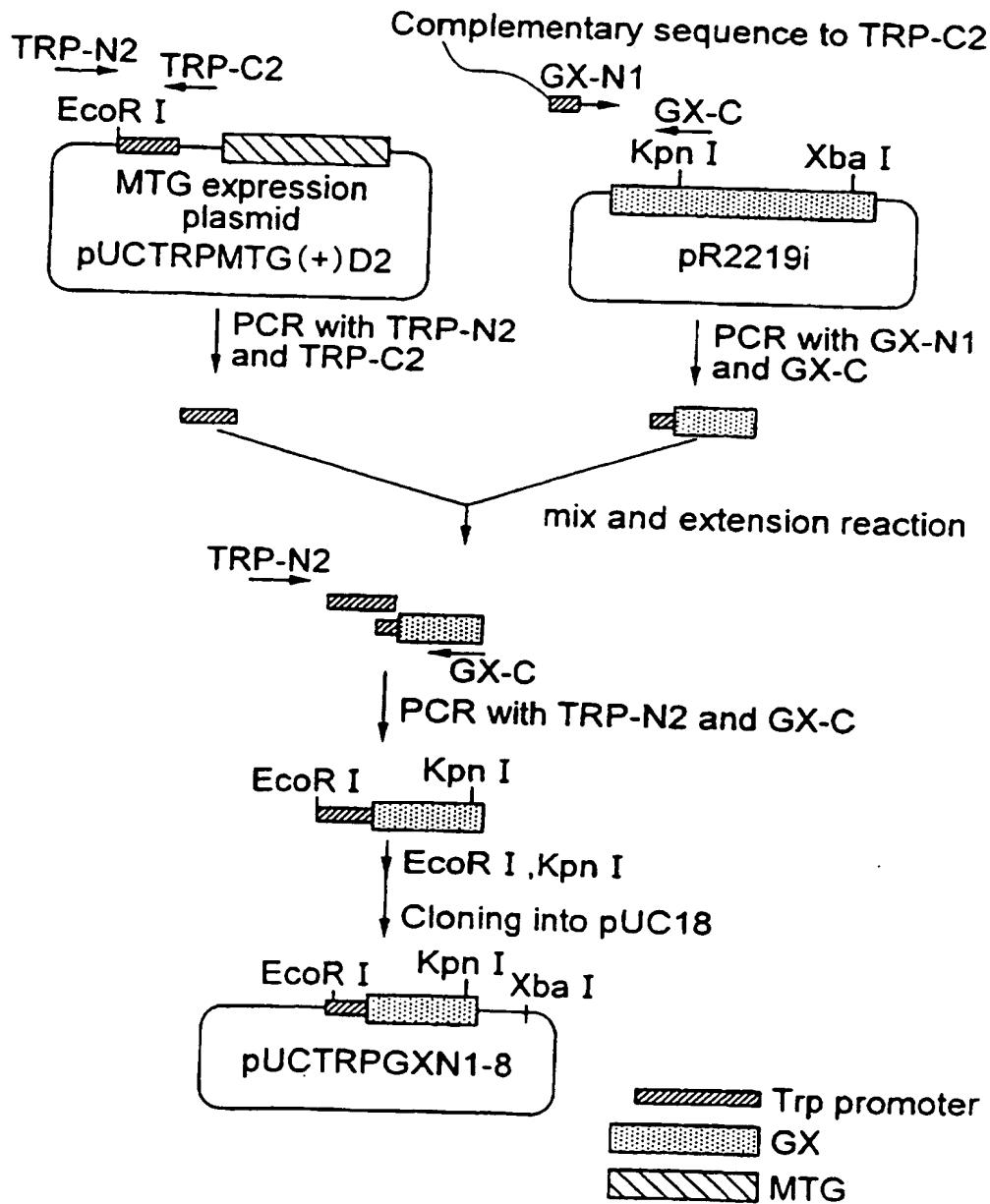


FIG. 2

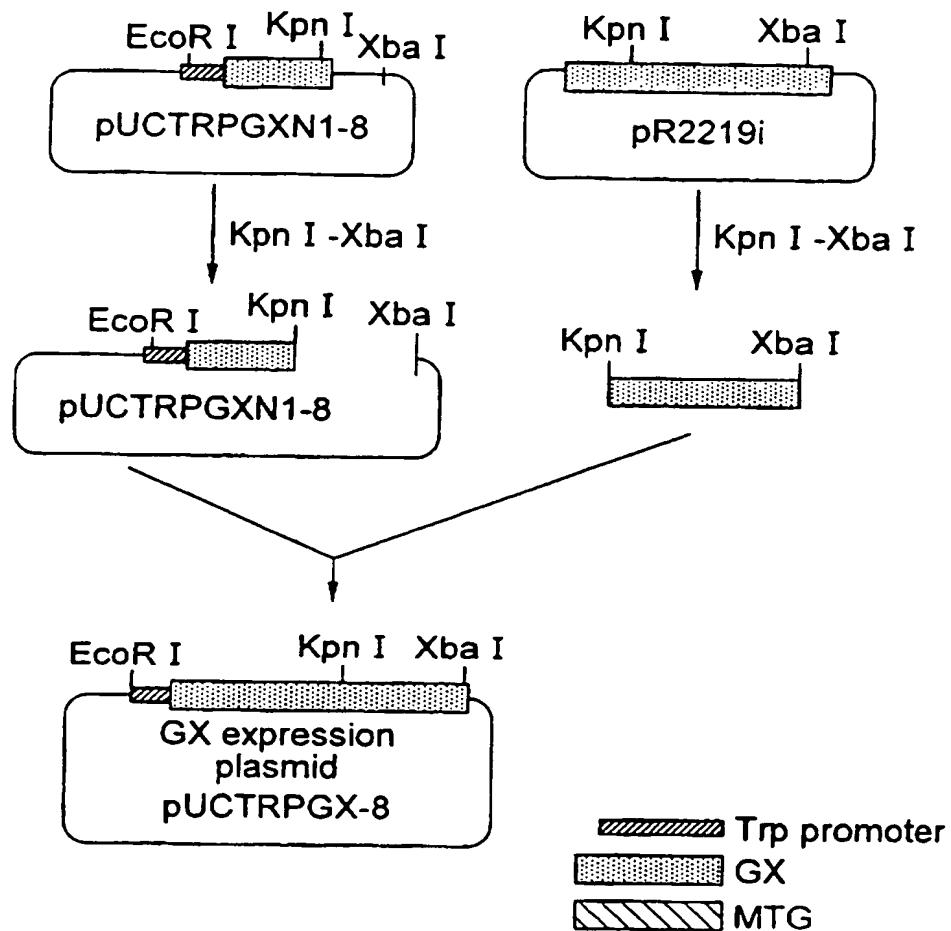


FIG. 3

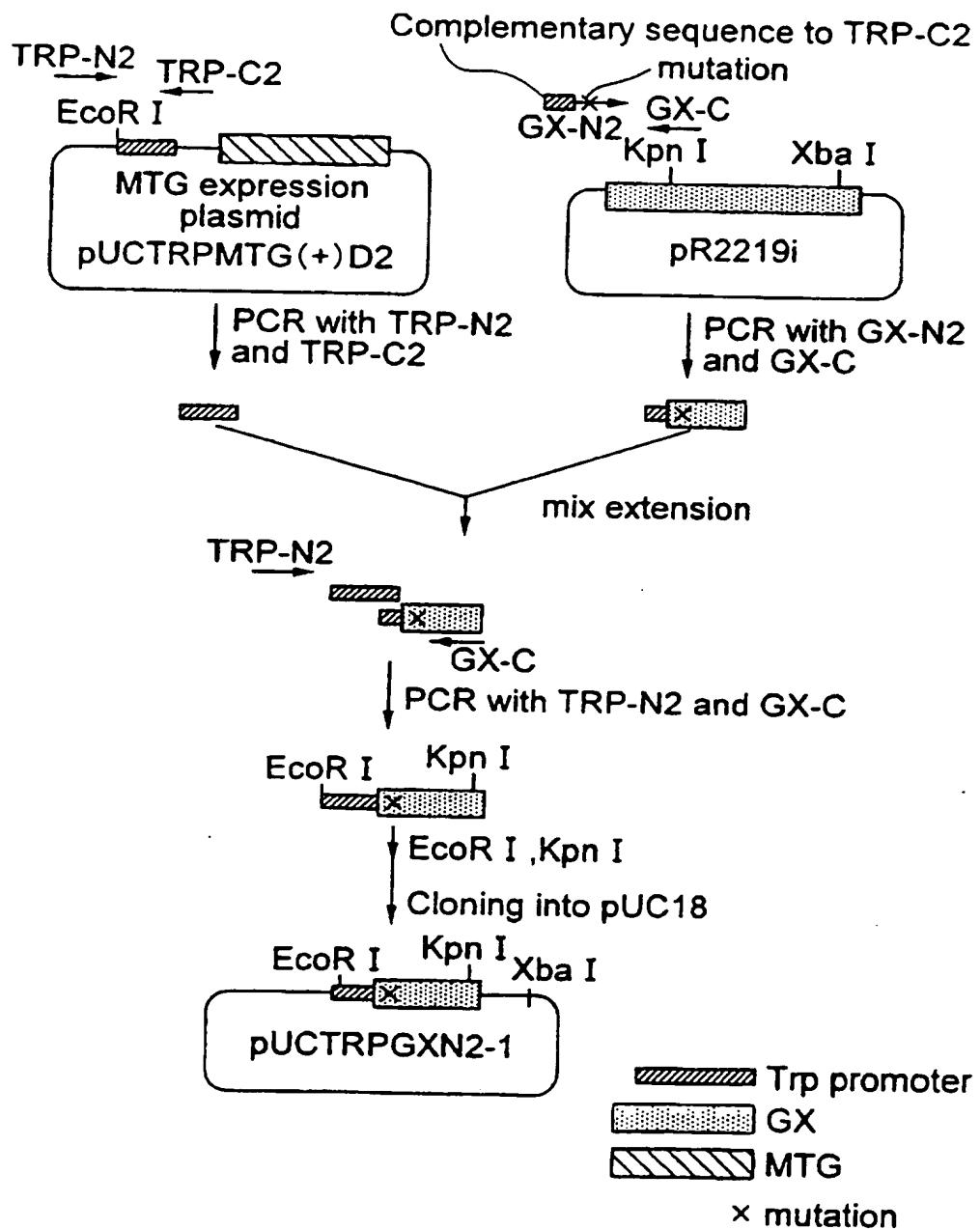
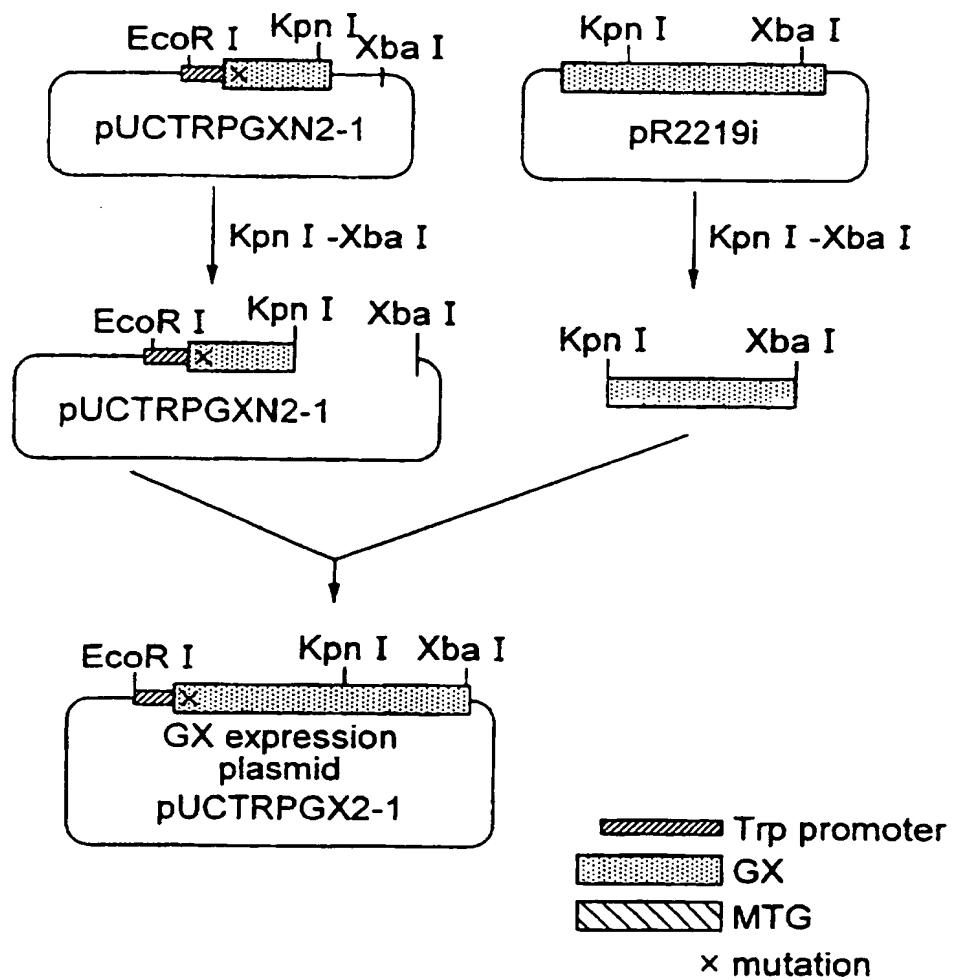


FIG. 4





European Patent
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EUROPEAN SEARCH REPORT

Application Number

EP 00 10 5313

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D, A	DATABASE EMBL NUCLEOTIDE AND PROTEIN SEQUENCES, 29 November 1993 (1993-11-29), XP002143207 HINXTON, GB AC = D24588. Rice cDNA, partial sequence (R2219_2A).EST. Oryza sativa * abstract *	1-9							
A	ASANO M ET AL: "Cysteine proteases from germinating soybean cotyledons, which contain 4-hydroxyproline." FASEB JOURNAL, vol. 11, no. 9, 1997, page A1223 XP000915491 17th International Congress of Biochemistry and Molecular Biology in conjunction with the Annual Meeting of the American Society for Biochemistry and Molecular Biology; San Francisco, California, USA; August 24-29, 1997 ISSN: 0892-6638 * abstract *	1-3, 18							
A	--- COUTON J M ET AL: "PURIFICATION AND CHARACTERIZATION OF A SOYBEAN COTYLEDON AMINOPEPTIDASE" PLANT SCIENCE (LIMERICK), vol. 75, no. 1, 1991, pages 9-17, XP000925433 ISSN: 0168-9452 * the whole document *	1-3, 18	C12N						
	---	-/-							
<p>The present search report has been drawn up for all claims</p> <table border="1"> <tr> <td>Place of search</td> <td>Date of completion of the search</td> <td>Examiner</td> </tr> <tr> <td>BERLIN</td> <td>24 July 2000</td> <td>Mateo Rosell, A.M.</td> </tr> </table>				Place of search	Date of completion of the search	Examiner	BERLIN	24 July 2000	Mateo Rosell, A.M.
Place of search	Date of completion of the search	Examiner							
BERLIN	24 July 2000	Mateo Rosell, A.M.							
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>									



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A	DATABASE EMBL NUCLEOTIDE AND PROTEIN SEQUENCES, 1 June 1998 (1998-06-01), XP002143209 HINXTON, GB AC = 036014. PROBABLE VACUOLAR AMINOPEPTIDASE I PRECURSOR (EC 3.4.11.22) (POLYPEP-TIDASE, LEUCINE AMINOPEPTIDASE-IV LAPIV, AMINOPEPTIDASE-III, AMINOPEPTIDASE YSCI. Schizosaccharomyces pombe. * abstract * ---	1-9	
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P, X	DATABASE EMBL NUCLEOTIDE AND PROTEIN SEQUENCES, 15 December 1999 (1999-12-15), XP002143210 HINXTON, GB AC= AW234700. Glycine max cDNA clone GENOME SYSTEMS CLONE ID: Gm-c1028-325 5' similar to TR:Q9Z2W0 Q9Z2W0 ASPARTYL AMINOPEPTIDASE, mRNA sequence. EST. From nt 380-870. * abstract * ---	1-9	TECHNICAL FIELDS SEARCHED (Int.Cl.7)
The present search report has been drawn up for all claims			
Place of search	Date of completion of the search	Examiner	
BERLIN	24 July 2000	Mateo Rosell, A.M.	
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone	T : theory or principle underlying the Invention		
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P : intermediate document	& : member of the same patent family, corresponding document		



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		CLASSIFICATION OF THE APPLICATION (Int.Cl.7)
		TECHNICAL FIELDS SEARCHED (Int.Cl.7)
T		1-9
The present search report has been drawn up for all claims		
Place of search	Date of completion of the search	Examiner
BERLIN	24 July 2000	Mateo Rosell, A.M.
CATEGORY OF CITED DOCUMENTS		
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : Intermediate document		
T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons S : member of the same patent family, corresponding document		

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EP 00 10 5313

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24-07-2000

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WO 9957274 A	11-11-1999	AU	3768199 A	23-11-1999

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- OTHER: Shading in drawings**

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